Oxidative stress and behavioral deficits in 3-nitropropionic acid-induced neurotoxicity in male mice: Neuroprotective effects of silymarin

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Research Article

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Abstract

Background

3-Nitropropionic acid (3-NP) is strongly believed to be a mitochondrial complex II irreversible inhibitor, leading to neural damages. Silymarin has been reported to exert various pharmacological manifestations including hepatoprotection, anti-inflammatory, anti-oxidant and cardioprotection. This study aimed to investigate neuroprotective features of silymarin against 3-Nitropropionic acid-induced neurotoxicity in male mice.

Methods

Six-week-old mice were received sub-chronic doses of 3-NP, intraperitoneally for 17 days. A group of mice were pretreated with silymarin (70 mg/kg/day, P.O.) for two weeks before 3-NP administration. Treatment group was received 3-NP for 17 days and then silymarin (70 mg/kg/day, P.O.) for 4weeks. At the end of the treatment schedule, animals were evaluated for behavioral alterations. Afterward, mice were sacrificed, neuronal damages in the hippocampus region of the brain tissues were performed by H&E staining, and the homogenates brain was used for evaluating the oxidative stress related parameters (Lipid peroxidation, nitric oxide, superoxide dismutase, glutathione, and total antioxidant capacity) and pro-inflammatory cytokine (TNF-α, IL-17, and IL-1β) levels.

Results

Our results indicated that 3-NP treatment significantly (p < 0.05) tends to reduce the motor coordination, memory, and neuronal antioxidant status while increased the pro-inflammatory cytokine levels. However, silymarin in both treatment and pretreatment protocols markedly (p < 0.05) attenuated the behavioral deficits, oxidative stress status, and neuroinflammation.

Conclusion

Results of the current study suggested that neuroprotective effect of silymarin against 3-NP-induced neurotoxicity might be due to mitigating oxidative stress status and provide insight into the therapeutic potential of silymarin.

1. Introduction

Mitochondrial malfunction and oxidative stress are two major risk factors in the pathophysiology and progression of neurodegenerative disease, including Huntington's disease (HD), Parkinson's disease (PD), Multiple sclerosis (MS), Alzheimer's disease (AD) (Colle et al. 2019). HD, as a dominantly inherited progressive neurological disorder, is characterized by movement disorder, cognitive impairment,
psychiatric and behavioral disturbance (Leegwater-Kim and Cha 2004). The striatum is the main pathologic site of HD. It has been reported that HD is caused by cytosine-adenine-guanine (CAG) repeat expansion in the mutant HTT gene, which increases the size of the polyglutamine tract in the N-terminal of the Huntington (Htt) protein. Although greatly controversial issues about neuronal death of the GABAergic medium spiny neurons (MSN) in the striatum are existing, mitochondrial dysfunction, bioenergetics defects, and following that oxidative stress are considered as the potent reason in the occurrence of this phenomenon (Phillips et al. 2008; Reddy and Shirendeb 2012).

3-Nitropropionic acid (3-NP) is a natural fungal nitro aliphatic compound, which is produced as a metabolite of 3-nitropropanol from various fungal species such as Aspergillus flavus, Astragalus, Arterionium and herbaceous plants (Brouillet et al. 2005; Comi et al. 2012). 3-NP intoxication is considered a popular model of HD-like syndrome in animals because of the production of selective striatal degeneration and mimics the progressive locomotor deterioration of HD (Tariq et al. 2005). Due to the ability of 3-NP to penetrate the blood-brain barrier (BBB), it acts as an irreversible inhibitor of succinate dehydrogenase (SDH), a mitochondrial complex II enzyme that is responsible for the oxidation of succinate to fumarate in Kreb's cycle (Cho and Kim 2015). On the other hand, like other parts of the body, the brain's cellular activities and neurons need adenosine triphosphate (ATP), which is produced mostly by mitochondria, and mitochondrial dysfunction can restrict the development of neurons. Notably, 3-NP interrupts oxidative phosphorylation, leading to decline the synthesis of ATP (Shalaby et al. 2018), alters calcium homeostasis (Gopinath et al. 2011), generation of reactive oxygen species (ROS), apoptosis and inflammation, neuronal damages and following that movement incoordination, increases anxiety, memory impairment, depression (Kumar et al. 2006) and significant decreases in body weight (Zhang et al. 2014).

Silymarin, as a mixture of polyphenolic flavonolignan, is extracted from the seeds of Silybum marianum plant (about 65–80%). Although silymarin is mostly used for the liver disorder (Federico et al. 2017), some advantages of silymarin can be included by crossing the BBB, no adverse effects in both humans and animals, good safety, and pharmacological actions such as anti-inflammatory, anti-oxidant, anti-cancer, cardioprotection, and neuroprotection (Haddadi et al. 2015; Haddadi et al. 2020b). Several experimental studies have been illustrated the neuroprotective effects of silymarin against neurodegenerative disease (AD and PD). For instance, silymarin markedly improved behavioral disorder and cell viability by inhibiting the Aβ fibrilization and deposition in an AD mouse Model (Murata et al. 2010). Also, it has been shown that silymarin, following neurotoxicity-induced damage, was able to inhibit the TLR4 pathway and subsequently decrease the elevated levels of TNF-α and NF-κB mRNA expression as well as decrease MDA content and increase the reduced levels of antioxidant factors, including SOD, CAT and GSH, which resulted in improving the memory and behavioral deficits (Ali et al. 2019; Haddadi et al. 2018). However, the present investigation was designed to study the neuroprotective effects of silymarin against sub-chronic administration of 3-NP-induced neurotoxicity.

2. Methods
2.1. Chemicals

Silymarin (PubChem CID: 7073228), 3-NP (PubChem CID: 160157), trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), n-butanol, 2,4,6-tripyridyl-S-triazine (TPTZ), ferric chloride, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma–Aldrich, St. Louis, USA. All other chemicals were of analytical grade and were obtained from Sigma Chemical CO.

2.2. Animals

Sixty healthy male mice (weigh 20–25 g) were purchased from the Laboratory of Animals Center of Hamadan University of Medical Sciences. The animals were acclimatized to the animal house conditions for one week before the experiments under constant environmental circumstances (25 ± 2°C and 55 ± 5% RH) of 12/12-h light/dark cycle with free access to standard food pellets and water. Animal studies were performed based on the guides of the National Institutes of Health for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the ethics committee of the Hamadan University of Medical Sciences (Res: IR.UMSHA.REC.1398.223).

2.3. Experimental design

At first, 3-NP was dissolved in distilled water and the pH was adjusted to 7.4. Silymarin suspended in CMC 0.5%. The animals were randomly divided in six groups of ten mice (Experimental groups are summarized in Table 1). The doses and protocol of administration of 3-NP and silymarin were selected based on previous studies (Cirillo et al. 2010; Yaghmaei et al. 2014). 24 h after last treatment, animals were subjected to behavioral studies. At the end of the last behavioral test, the animals body weight was measured and then anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Subsequently, animals sacrificed to collect their brain samples and preserved at -80°C for biochemical and the levels of neuroinflammatory markers analysis. Experimental procedure is depicted on the Fig. 1.

Table 1. Drug dose and route of administration of groups.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Experimental design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>saline solution</td>
</tr>
<tr>
<td>Sham</td>
<td>solvents of silymarin and 3-NP</td>
</tr>
<tr>
<td>3-NP</td>
<td>Twice a day sub-chronic injection of 3-NP for 17 days were divided as follows: 7.5 mg/kg for the first 2 days of treatment, followed by 3.75 mg/kg for 7 days, finally a dosage of 2 mg/kg for the last 8 days of treatment.</td>
</tr>
<tr>
<td>Treatment</td>
<td>3-NP (sub-chronic administration for 17 days) + silymarin (70 mg/kg/day P.O. from the 18th day for 4 weeks)</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Silymarin (70 mg/kg/day P.O.) for two weeks and then 3-NP sub-chronic administration</td>
</tr>
<tr>
<td>Silymarin</td>
<td>silymarin (70 mg/kg/day P.O.) for 4 weeks</td>
</tr>
</tbody>
</table>

Abbreviations: 3-NP; 3-Nitropropionic acid, i.p; Intraperitoneally, P.O.; Per oral.

### 2.4. Measurement of body weight

The body weight of mice was measured prior to 3-NP administration (1st day) and on the last injection day. The percentage change in body weight was calculated as follows:

\[
\text{Change in body weight} = \frac{\text{body weight (1st day)} - \text{the last injection day}}{\text{1st day body weight}} \times 100.
\]

### 2.5. Behavioral studies

#### 2.5.1. Rotarod test

As one of the best behavioral tests to evaluate motor coordination and balance, the rotarod is a suitable and sensitive test in detecting cerebellar dysfunction in rodents. and is especially sensitive. However, motor coordination, learning and cardiopulmonary endurance can affect the motor performance on the rotarod test. The procedure was performed according to the method described previously (Haddadi et al. 2020a; Shiotsuki et al. 2010). Briefly, the rotating rod device consists of a wheel whose rotational speed varies between 0-40 rpm. The wheel is about 20 cm away from the ground and is divided into 4 separate parts by spherical plates. Also, the wheel was covered with hard chloroethylene, which does not permit gripping on the surface. In this test, the training session was performed for two days at 5, 8, 16 and 24 rpm (each mouse for 5 minutes). However, each mouse was allowed to rest for 1 hour between different RPMs. On the test session, all mice were tested at 3 to 25 rpm and the time of fall was recorded. The animal was placed back on the wheel immediately after falling, up to 5 times.

#### 2.5.2. Open field test (OFT)

The open field activity monitoring system make it easy to comprehensively assesses locomotor, anxiety like, and exploratory behavioral activity levels in animal models of neuromuscular disease and efficacy of
therapeutic drugs that may enhance locomotion and muscle function. The protocol of test was performed according to the method described by Tatem et al (Tatem et al. 2014). In summary, after habituation session, each mouse was placed in the empty open field box (50×50×45 cm³) and allowed to explore for five minutes. Finally, behavioral activities were evaluated by using computer software connected to the box. The results of this test reported as total number of crossings, total traveled distance (cm), mean velocity (cm/s), number of crossing in the center, and distance in center (cm).

2.5.3. Forced swim test (FST)

The forced swimming test (FST) is one of the most valid and common tests for depression in rodents. The test method is designed based on a cylindrical container (45×30×79 cm) made of acrylic glass filled with water at 25-23 °C and the animal is gently placed in it. The duration of animal swimming is 6 minutes and the duration of immobility (non-swimming) is calculated from the second minute. Conventionally, the cessation of movement of the mouse's limbs and its floating is considered as immobility and its duration is considered as immobility time (s) (Castagné et al. 2010).

2.5.4. Tail suspension test (TST)

The tail suspension test was developed by an Italian researcher to measure despair. In this method, the mouse is hung from a tail in a room. Usually after hanging, healthy mice begin to struggle and paddle to escape their suspension position. The more depressed the rat is, the less it will try to escape the suspension. The duration of this test was 6 minutes and the immobility time was calculated from the second minute. The length of time that the animal was suspended in the air was considered as the immobility time (Zavvari and Karimzadeh 2017).

2.5.5. Elevated plus maze test (EPM)

Elevated plus maze (EPM) is a behavioral test to assess anxiety and memory retention in rodents. The apparatus is placed 60 cm above the floor. In training phase, during which the mice were placed at one end of the open arm (50×10 cm) and the position of each mouse was in such a way that its face was opposite to the central square. Each mouse was allowed to move freely about the maze for five minutes. To monitor working memory performance, 24 hours after the training, the test phase was conducted by placing each mouse at one of open arms and the time takes the mouse to enter into any of the closed arms (50×10×20 cm) with all its four paws was recorded by a stopwatch as transfer latency (Walf and Frye 2007).

2.5.6. Passive avoidance test (PAT)

Passive avoidance is commonly used to evaluate short-term or long-term memory of animals. The testing apparatus and the procedure of the test was performed the same as previously described (Mohammadi et al. 2020). PAT consists of two phases: training and test phases. For training, the mice were placed in a light compartment to explore freely for 30 seconds. The door was opened and the moment mice entered
the dark compartment with all its four paws, the door closed and a 1-2 second foot shock (0.5 mA) was delivered via the rods and after ten Sec mice moved back into their home cage. The training phase was repeated after 30 min. After one day, during the test phase mice were again placed inside the light compartment and the time takes to enter into the dark compartment was recorded as step through latency (STL). The cut-off time for the test phase was 180 Sec. STL A (evaluated STL on the training day) and STL R (evaluated STL on the test day) were assessed after 30 min and 24 h after the training phase, respectively.

2.6. Biochemical tests

Brains having homogenized in cold phosphate buffered saline (10% w/v), the suspension was centrifuged at 12000 xg for 15 min at 4 °C and supernatant was removed for biochemical analysis. Lipid peroxidation (LPO) was determined using thiobarbituric acid reactive substances (TBARS) method to estimate the amount of malanodialdehyde (MDA) produced by absorbance reading in 532 nm (Makhdoomi et al. 2020) and the results were reported as nmol/mg protein. Brain nitric oxide (NO) was estimated using Griess reagent (1% sulfanilamide, 0.1% NED, and 2.5% phosphoric acid) by absorbance reading in 520 nm (Nili-Ahmadabadi et al. 2018) and the results were expressed as µmol/mg protein. Total antioxidant capacity (TAC) was assessed by reducing Fe^{3+} to Fe^{2+} in the presence of TPTZ. The absorption of the blue complex reading in 593 nm (Zeinvand-Lorestani et al. 2018) and the results were reported as µmol/mg protein. In addition, Total thiol molecule (TTM) was assessed using DTNB as the reagent, reacts with the thiol groups to yield a yellow-colored compound by maximum absorption at 412 nm (Pourkhalili et al. 2011) and the results were expressed as mmol/ mg protein. The last, superoxide dismutase (SOD) activity was determined according to its kit protocol (Navand Salamat Co, Iran) and the results were reported as U/mg protein. At the end of each experiment, protein content was measured in the crude homogenate of brain according to the Bradford method using bovine serum albumin (BSA) as a standard.

2.7. Determination of TNF-α, IL-17, and IL-1β levels

The levels of interleukin (IL)-1β, IL-17, and tumor necrosis factor (TNF)-α in the brain homogenates were measured using a monoclonal antibody-based ELISA kit (Bio legend, USA) according to the manufacturer’s instructions as previously described (Kheradmand et al. 2016). The concentrations of the pro-inflammatory cytokine were expressed in pg/100mg wet tissue.

2.8. Histological studies

After separation, brain (n=3/group) tissues were immediately submerged in 10% neutral buffered formalin solution. Brain tissues were dehydrated in graded concentrations of alcohol, cleared in xylene, and finally embedded in paraffin. Sections (five-micron thickness) fixed on clean slides and stained with hematoxylin and eosin (H&E). Continually, the histological variations and the number of healthy pyramidal cells were studied under a light microscope at 40x magnification by using a digital camera (Nikon E800, Japan) attached to a microscope and image j software (Mohammed and Mansour 2021).
2.9. Statistical analysis

Results were expressed as the mean±standard error of the mean (SEM) and analyzed by Graph Pad Prism software, version 6.0. The statistical difference of the values was determined by one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons. The $P$ value of $<0.05$ was considered statistically significant.

3. Results

It should be noted that no significant changes were seen in silymarin and sham groups.

3.1. Effect of Silymarin on body weight changes in 3-NP-treated animals

Body weight changes is depicted in Fig 2. Mice were treated 3-NP showed significantly decreased body weigh in during treatment as compared to the control group ($p<0.01$). Silymarin pretreatment significantly ($p<0.01$) harnessed weight loss in mice and treatment with silymarin markedly decreased weight loss in comparison with the 3-NP group.

3.2. Effect of Silymarin on fall latency in rotarod test in 3-NP-treated animals

As shown in Fig 3, the fall latency significantly ($p<0.001$) decreased in 3-NP group as compared to control group. However, pretreatment with silymarin significantly ($p<0.001$) prevented the motor incoordination. Fall latency levels of treatment group found to be increased significantly ($p<0.05$) compared to the 3-NP group.

3.3. Effect of Silymarin on open field test in 3-NP-treated animals

Depicted in Fig 4 is the evaluated parameter in the open field test. First of all, total number of crossings decreased significantly ($p<0.05$) in 3-NP group in comparison with control group. Treatment and pretreatment with silymarin caused a significant increase ($p<0.05, p<0.01$, respectively) compared to the 3-NP group (Fig 4A). However, administration of 3-NP led to decrease the total traveled distance ($p<0.01$) as compared to control group, while silymarin in both treatment and pretreatment groups significantly ($p<0.05, p<0.01$) improved the total traveled distance in comparison with 3-NP group (Fig 4B). The mean velocity levels in 3-NP group was significantly ($p<0.001$) lower in comparison with control group. Treatment and pretreatment with silymarin were able to significantly ($p<0.001$) enhance the mean velocity levels as compared to the 3-NP group (Fig 4C). As shown in Figure 4d and e, there is seen to a marked ($p<0.001$) decrease of the number of crossings in center and the distance in center in the 3-NP group. Notably, pretreatment with silymarin prevented the reduction in exploratory behavioral activity and silymarin treatment led to significantly increase in the number of crossings in center ($p<0.05, p<0.01$) and the distance in center ($p<0.001$) as compared to the 3-NP group (Fig 4d, 4E).

3.4. Effect of Silymarin on immobility time in FST and TST in 3-NP-treated animals
The data revealed from immobility time indicated that 3-NP administration caused depression because of the significant increase \((p<0.001)\) in immobility time compared with the control group. However, pretreatment with silymarin was able to attenuate \((p<0.01)\) depression and silymarin treatment significantly \((p<0.05)\) decreased the immobility time in comparison with the 3-NP group (Fig 5A, 5B).

### 3.5. Effect of Silymarin on transfer latency in EPM test in 3-NP-treated animals

According to results, in 3-NP group significant \((p<0.001)\) increase in transfer latency was seen as compared to the control group. However, pretreatment with silymarin significantly \((p<0.01)\) improved memory retention which indicated by the transfer latency in mice. Furthermore, the transfer latency in mice that treated with silymarin markedly decreased \((p<0.05)\) in comparison with the 3-NP group (Fig 6).

### 3.6. Effect of Silymarin on step through latency in PAT in 3-NP-treated animals

The obtained results from PAT showed that 3-NP caused a significant reduction in step through latency in STL R \((p<0.001)\) as compared to the control group. The long-term memory deficits significantly \((p<0.001)\) prevented by silymarin in pretreatment mice group. However, the STL R was found to be significantly \((p<0.01)\) increased in treatment group compared to the 3-NP group (Fig 7). No significant changes were observed in STL A.

### 3.7. Oxidative Stress status

Table 2 illustrates the alternations of oxidative stress biomarkers.

The MDA and NO levels significantly \((p<0.001)\) increased in 3-NP group as compared to the control group. Silymarin pretreatment significantly inhibited the elevation in MDA and NO levels \((p<0.001, p<0.01, \text{respectively})\) in comparison with 3-NP group. Also, silymarin treatment was able to decrease the MDA and NO levels significantly \((p<0.05)\) as compared to the 3-NP group. Based on revealed data, the TTM, SOD activity, and TAC levels were found to be significantly \((p<0.001)\) decreased in comparison with control group. Moreover, pretreatment with silymarin markedly \((p<0.001)\) prevented the reduction in the TTM, SOD activity, and TAC levels due to 3-NP in mice. The TTM, SOD activity, and TAC levels significantly \((p<0.01)\) increased in mice that received silymarin as compared to the 3-NP group.

**Table 2** Effect of Silymarin on oxidative stress status in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group.
<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/mg protein)</th>
<th>NO (µmol/mg protein)</th>
<th>TTM (mmol/mg protein)</th>
<th>SOD activity (U/mg protein)</th>
<th>TAC (µmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.3±0.71</td>
<td>6.1±0.68</td>
<td>3.9±0.43</td>
<td>241±10.73</td>
<td>0.41±0.05</td>
</tr>
<tr>
<td>Sham</td>
<td>6.1±0.68</td>
<td>5.9±0.72</td>
<td>4.1±0.57</td>
<td>240±7.42</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>3-NP</td>
<td>12.8±1.6***</td>
<td>13.4±2.1***</td>
<td>1.4±0.19***</td>
<td>124±4.76***</td>
<td>0.23±0.04***</td>
</tr>
<tr>
<td>Treatment</td>
<td>10.1±1.7#</td>
<td>10.6±1.3#</td>
<td>2.1±0.33##</td>
<td>196±5.44##</td>
<td>0.28±0.06##</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>8.3±0.94###</td>
<td>8.1±1.03###</td>
<td>2.9±0.37###</td>
<td>213±8.13###</td>
<td>0.36±0.05###</td>
</tr>
<tr>
<td>Silymarin</td>
<td>5.6±0.62</td>
<td>5.2±0.63</td>
<td>4.2±0.48</td>
<td>245±9.61</td>
<td>0.45±0.03</td>
</tr>
</tbody>
</table>

3.8. Effect of Silymarin on pro-inflammatory cytokines in 3-NP-treated animals

To further explore on the protective effects of silymarin against 3-NP-induced neurotoxicity, the potential effect of silymarin on the level of IL-1β, IL-17, and TNF-α was investigated in the literature. Data presented in Fig 8 showed that sub-chronic 3-NP administration significantly (p<0.001) elevated levels of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-17 as compared to the control group. The elevation in TNF-α, IL-1β, and IL-17 levels were significantly (p<0.01) prevented in mice pretreated with silymarin in comparison with the 3-NP group. Furthermore, treatment with silymarin markedly (p<0.05) decreased the inflammatory markers as compared to the 3-NP group (Fig 8A, 8B, 8C).

3.9. Effect of Silymarin on hippocampal pyramidal cells in 3-NP-treated animals

As shown in Fig. 9, 3-NP caused a significant (p<0.001) decrease in the number of hippocampus pyramidal cells in comparison with the control group. Notably, treatment with silymarin lead to a marked (p<0.05) improve in the number of intact cells in CA1 region and silymarin pretreatment (p<0.01) prevented neuronal degeneration which is indicated by the high number of hippocampus healthy pyramidal cells as compared to the 3-NP group.

3.10. Effect of Silymarin on histological alterations

Microscope examination of the hippocampus Cornu Ammonis (CA1) region of control, sham, and silymarin groups (Fig. 10A, B, F, respectively) revealed that the pyramidal cells have clear and transparent structures with large nuclei, and peripheral distribution of nissl granules. On the other hand, darkly stained nuclei, pyknotic nuclei, and wide shrinkage in pyramidal cells in the 3-NP-treated mice are indication of neurodegeneration (Fig. 10C). Although silymarin treatment (Fig. 10E) made a moderate amelioration of neuronal degeneration accompanied by increasing in the intact pyramidal cells, silymarin pretreatment (Fig. 10D) markedly was able to prevent neurodegeneration induced by 3-NP which is indicated by the normal morphology and healthy neurons of pyramidal cells, decrease pyknotic nuclei, and normal microglial cells in hippocampal CA1 region.
4. Discussion

3-Nitropropionic acid-induced neurotoxicity is characterized by behavioral alterations, memory impairment, significantly increased oxidative stress, and pro-inflammatory cytokine induction. Our results of this study showed that not only did 3-NP lead to increase the movement incoordination, depression, and memory dysfunction in behavioral tests, but also, 3-NP caused significant lipid peroxidation and diminished enzymatic and non-enzymatic antioxidant activities in the brain by decreasing levels of SOD, TAC, as well as TTM and following that increasing pro-inflammatory-related cytokine (IL-1β, IL-17, and TNF-α). On the other hand, pretreatment with silymarin in sub-chronic 3-NP-exposed mice prevented the elevation in motor incoordination, memory impairment, depression, MDA levels, NO levels, and pro-inflammatory markers. Added to these, there is seen to be a significant amelioration in behavioral deficits, oxidative stress biomarkers, and pro-inflammatory markers in mice treated with silymarin indicated with increasing antioxidant enzymes activity.

It has been approved that 3-NP is a mitochondrial toxin because of its influence on mitochondria by irreversible inhibiting of SDH, a respiratory chain complex II enzyme, and thereby inhibits the TCA cycle energy metabolism to produce ATP (Beal et al. 1993; Kumar and Kumar 2009). Due to the intense energy requirement for neurons to maintain ion gradients across the plasma membrane, mitochondrial dysfunction can lead to neuronal death (Túnez et al. 2010). Although there are some suggestions about the 3-NP neurotoxicity mechanism, in total, 3-NP induce neurotoxicity via at least three mechanisms: a) increasing the intracellular levels of Ca^{2+}, in turn, causing glutamate release and excitotoxicity (Kumamoto et al. 2014), b) mitochondrial dysfunction (Kumar and Kumar 2009), c) free radical generation and oxido-nitrosative stress (Gao et al. 2015), and all these phenomena result in neuronal damages.

To the best of our knowledge, this is the first time that silymarin has been investigated as potential agent against 3-NP-induced neurotoxicity. 3-NP induced body weight loss which might be related to factors such as impairment in energy metabolism, bradykinesia and striatal lesions in the animals (La Fontaine et al. 2000). However, pretreatment with silymarin markedly inhibited weight loss in experimental groups. Sub-chronic administration of 3-NP resulted in anomalies of movement and motor performance and pretreatment with silymarin significantly prevented the elevation motor incoordination and balance. It should be noted that motor incoordination is related to basal ganglia lesions of the brain and silymarin ameliorated the motor and movement disorders in 3-NP-induced striatal lesions in the brain of mice (Ahuja et al. 2008; Guyot et al. 1997). Also, as seen in the open field test, 3-NP caused to decrease in motivation to move or to explore the environment which was evidenced by evaluated parameters. As a suitable test to measure anxiety and depression, in FST and TST, it was found that administration of 3-NP induces symptoms like disappointment, muscular weakness and depression, indicated by increasing immobility time. As the index for memory impairment and cognitive deficit, transfer latency and step through latency increased markedly in the 3-NP group in EPM and PAT tests, respectively. These results endorse that not only does 3-NP neurotoxicity cause neural death in the striatum but also 3-NP leads to cognitive dysfunction and severe devastation to the neurons present in other regions of the brain like the
hippocampus and hippocampal CA1 and CA3 pyramidal neural injuries (Kumar and Kumar 2009; Ludolph et al. 1991). Totally, sub-chronic doses of 3-NP could lead to the development of behavioral alterations and cognitive deficits and characteristics of advanced HD phases. Several investigations have been demonstrated that the animals that were treated with sub-chronic 3-NP administrations decreased locomotor activity, movement disorders/or motor incoordination, marked gait abnormalities, significant depression, and memory deficits (Antunes et al. 2021; Danduga et al. 2018; Hariharan et al. 2014; Mu et al. 2011; Pandey et al. 2008; Picconi et al. 2006), which these results endorse our finding in the literature. On the other hand, silymarin treatment and pretreatment showed improvements since this flavonoid mitigated motor and cognitive impairments that were induced by the 3-NP treatment, as were determined by the several cognitive and motor behavioral tests. The present study showed that silymarin pretreatment had more protective effects in mice in comparison with treatment group in all behavioral studies. Silymarin could improve motor coordination and exploratory activity, as seen in the rotarod and open field test. Besides, the improvement in depression, anxiety, and spatial memory, which indicated by immobility time and transfer latency, respectively, were observed with the silymarin treatment and especially pretreatment in mice that received 3-NP. In an experimental study, Antunes et al. (2021), demonstrated that the CTK 01512-2 (a recombinant peptide, which is derived from the spider venom) treatment prevented motor impairments, anxious behavior, and spatial memory impairment that were induced by the 3-NP in rats (Antunes et al. 2021).

As mentioned above, mitochondrial dysfunction and following that oxidative stress are two main neurotoxicity mechanisms of 3-NP. It has been reported that administration of 3-NP is associated with mitochondrial dysfunction, ATP depletion, increased influx of Ca$^{2+}$, ROS generation, and oxidative stress (Shalaby et al. 2018). As stated, 3-NP is an irreversible inhibitor of SDH, resulting in ATP depletion and anomalies of the intracellular Ca$^{2+}$, in turn, activating oxidative damages and excitotoxicity, and subsequently neuronal cell death (Maya-López et al. 2017). The excitotoxicity is might be due to the Na$^+$/K$^+$-ATPases dysfunction, and gradually depolarization of the neuronal cells, resulting in the glutamate receptor activation of the NMDA (Liot et al. 2009). Furthermore, oxidative stress is strongly believed to play a crucial role in 3-NP-induced neurotoxicity. Herein, our data precisely has shown that 3-NP significantly increased the MDA and NO levels in the brain mice, while the level of enzymatic and non-enzymatic antioxidants such as SOD, TAC, and TTM was decreased. Several studies have been demonstrated that 3-NP led to decreased succinate dehydrogenase and GSH levels, and increased the MDA, NO, and ROS levels in rats (Dhadde et al. 2016; Kumar et al. 2007). Also, in a model of HD in rats, Túnez et al. (2004), reported that 3-NP administration caused to decrease the succinate dehydrogenase activity and increased the MDA level in the brain striatum (Túnez et al. 2004). As the main characteristic feature of oxidative stress, lipid peroxidation can be occurred by degradation of polyunsaturated acids in the cell membrane due to excessive production of ROS. Following administration of 3-NP increased NO can lead to nitrosative stress. The reaction between NO and superoxide anion produces Peroxynitrite (ONOO$^-$) which can damage unsaturated fatty acids in the cell wall and intensify lipid peroxidation (Korkmaz and Kolankaya 2013). In this regard, silymarin showed an inhibitory effect on oxido-nitrosative stress by decreasing the MDA and NO levels, which is in agreement with the previous findings (Haddadi et
Antioxidant enzymes, such as SOD, TAC, and GPx, are considered to be the first line of cellular defense to inactivate ROS and inhibit ROS-induced deterioration (Ighodaro and Akinloye 2018). Among these, the enzyme of SOD catalytically converts the superoxide radical anions into hydrogen peroxide ($\text{H}_2\text{O}_2$) and oxygen molecules, following that GPx can use thiol molecules such as glutathione, as a reductant, to minimize the destructive effects of $\text{H}_2\text{O}_2$. According to our results, reduced SOD activity might be related to excessive generation of superoxide anions because of mitochondrial dysfunction induced by 3-NP. However, pretreatment with silymarin significantly ameliorated antioxidant status and oxidative stress, which was evidenced by increasing SOD, TAC, and TTM levels in the brain mice. Also, increased TTM levels in treatment groups are associated with increased production of active thiols such as glutathione. Several studies illustrated that antioxidant features of silymarin may be related to effectively inhibiting ROS production and nitric oxide pathway (Borah et al. 2013; Haddadi et al. 2018; Raza et al. 2011).

Added to these, 3-NP administration let to marked activation of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β, which are released by activated microglia in striatum (Sawada et al. 2006). In this regard, our results evidenced that 3-NP significantly increased pro-inflammatory markers, which is in agreement with previous studies that revealed similar pro-inflammatory cytokines enhancement following administration of 3-NP (Bhateja et al. 2012; Jamwal and Kumar 2016). It has been reported that excessive release of pro-inflammatory cytokines directly activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), in turn, leading to an increased production of NO and eventually neuron degeneration (Drew and Chavis 2000). Conversely, pretreatment with silymarin markedly prevented enhanced levels of TNF-α, IL-6 and IL-1β with silymarin treatment decreasing the elevated pro-inflammatory markers due to 3-NP-treated mice. Anti-inflammatory features of silymarin exerted by modulating the pro-inflammatory cytokines (IL-1β, IL-17 and TNF-α) level and probably up-regulated percentage of NF-κB mRNA expression and reduction of oxido-nitrosative stress (Ali et al. 2019; Haddadi et al. 2014; Moghaddam et al. 2020).

Furthermore, the number of healthy pyramidal cells in the hippocampus CA1 region in the 3-NP group approximately was a quarter as many as that in the control group. However, superior neuroprotective benefits of silymarin pretreatment beyond that of silymarin treatment were observed by increasing the number of intact neurons in the CA1 region. In a model of HD-like symptoms in rats, it has been demonstrated that 3-NP treatment caused a marked neuronal and neurofibrillary degeneration and decreased the viable pyramidal cells count in the hippocampus CA1 region of the brain (Danduga et al. 2018), which is in line with the literature. Moreover, Sugino et al, showed that hippocampal CA1 and CA3 regions are vulnerable in administration of 3-NP which is indicated by neuronal degeneration, such as cell shrinkage and pyknotic nuclei (Sugino et al. 1999). However, silymarin pretreatment was superior to silymarin treatment to prevent and enhance the normal morphology of the hippocampus pyramidal cells.

**Conclusion**
In conclusion, our findings indicated that silymarin pretreatment was able to prevent 3-NP-induced neurotoxicity. Neuroprotective potential effects of silymarin could be demonstrated by improving free radical scavenging and increasing the enzymatic and non-enzymatic antioxidant levels accompanied by decreasing the elevated levels of MDA and NO against 3-NP-treated mice. Furthermore, pretreatment with silymarin markedly prevented the elevation of pro-inflammatory markers and neuronal degeneration. In addition, there is seen to be a marked improvement in behavioral deficits by increasing motor coordination, depression, and memory function with silymarin-treated mice. Taken together, silymarin pretreatment had better improvement than silymarin treatment to prevent brain damage after the 3-NP challenge. Also, this study can provide insight into the therapeutic potential of silymarin treatment against 3-NP intoxication but further study is needed to investigate the pharmacological significance of silymarin effects on brain neurotoxicity.

**Declarations**

Conflicts of interest and source of funding: None declared

**Ethical Approval**

All animal studies were performed based on the guides of the National Institutes of Health for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the ethics committee of the Hamadan University of Medical Sciences (Res: IR.UMSHA.REC.1398.223).

**Consent to publish**

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

**Authors' contributions**

SEB (Ph.D. of neuroscience) was carried out in preparation of histological slides and statistical analyzes and drafting. SM (MS of Toxicology) participated and was involved in the interpretation of data, final check of the draft and helping to revise the manuscript. AF (MS of Toxicology) is involved in biochemical analyzes and drafting. AD (MS of Toxicology) is involved in biochemical analyzes. AK (Ph.D, Prof. of Physiology) was involved in support of the study and final checking of the manuscript. RH (Ph.D, A/Prof. of Pharmacology), the supervisor of the study, was involved in the concept, design, support of the study, drafting and final checking of the manuscript. All authors read and approved the final manuscript.

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**Availability of supporting data**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Disclosure statement**

The authors declare that they have no conflict of interest.

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Figures
Figure 1

Schematic representation of the experimental procedure. A) pretreatment group procedure, B) treatment group procedure

Figure 2

Effect of Silymarin body weight changes in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol
Figure 3

Effect of Silymarin on fall latency in rotarod test in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol.
Figure 4

Effect of Silymarin on open field test in 3-NP-treated animals. (A) total number of crossing, (B) total traveled distance, (C) mean velocity, (D) number of crossing in center, (E) distance in center. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol

Figure 5

Effect of Silymarin on immobility time in FST in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). (A) immobility time in FST, (B) immobility time in TST. *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol
Figure 6

Effect of Silymarin on transfer latency in EPM test in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol.

Figure 7

Effect of Silymarin on transfer latency in EPM test in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ###p<0.001 as compared to 3-NP group.
##\ p<0.01, \text{ and } ###\ p<0.001 \text{ as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol.

Figure 8

Effect of Silymarin on pro-inflammatory cytokines in 3-NP-treated animals. Data are expressed as mean±SEM (n=10). (A) IL-1β, (B) IL-17, (C) TNF-α. *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol.
Figure 9

Effect of Silymarin on hippocampal pyramidal cells. Data are expressed as mean±SEM (n=10). *p<0.05, **p<0.01, and ***p<0.001 as compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 as compared to 3-NP group. T: treatment protocol, pre T: pre-treatment protocol.
Figure 10

Hematoxylin-eosin staining of hippocampus Cornu Ammonis 1 (CA1) region of animals at 40x magnification. Groups: Control (A), Sham (B), 3-NP (C), Silymarin+3-NP (preT) (D), Silymarin+3-NP (T) (E), Silymarin (F)